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Chapter 4

Mouse DC-SIGN/CD209a as target for antigen delivery and adaptive immunity

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Mouse DC-SIGN/CD209a as target for antigen delivery and adaptive immunity

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The efficacy of vaccination studies aimed at targeting antigens to human DC-SIGN have been notoriously difficult to study *in vivo*, as eight DC-SIGN homologues have been described in mice. CD209a/SIGNR5 has been coined as the murine DC-SIGN (mDC-SIGN) ortholog, based on its expression and location in the genome. Nonetheless, which properties of human DC-SIGN are covered by mDC-SIGN is poorly investigated. One of the most important functions of DC-SIGN is the induction of adaptive immunity. As such, the aim of this study is to determine the capability of mDC-SIGN to induce adaptive immune responses. Here, we show that mDC-SIGN is expressed on GM-CSF cultured bone marrow-derived dendritic cells and macrophages. However, unlike hDC-SIGN, mDC-SIGN is an internalizing receptor which quickly resurfaces after internalisation. Binding of OVA-coupled anti-mDC-SIGN antibody by BMDCs leads to quick internalization, processing and presentation to antigen-specific CD8⁺ and CD4⁺ T cells, which can be boosted using the TLR4 ligand, MPLA. In the homeostatic condition, mDC-SIGN is mostly expressed on myeloid cells in the skin and spleen. A subcutaneous injection of fluorescently-labeled anti-mDC-SIGN reveals specific targeting to mDC-SIGN⁺ skin dendritic cells and monocyte-derived dendritic cells *in situ*. A subcutaneous vaccination strategy containing OVA-coupled anti-mDC-SIGN antibody generated antigen-specific polyfunctional CD8⁺ T cell and CD4⁺ T cell responses and a strong isotype-switched OVA-specific antibody response *in vivo*. We conclude that mDC-SIGN shows partly overlapping similarities to hDC-SIGN and that targeting mDC-SIGN provides a valuable approach to investigate the immunological function of DC-SIGN *in vivo*.

Introduction

The human innate immune receptor DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin) recognizes pathogen- and host-derived glycoproteins(1). Additionally, it mediates antigen internalization, processing and presentation of antigens to T cells, which are functional hallmarks of antigen presenting cells (APCs). Therefore, targeting antigens to human DC-SIGN (hDC-SIGN) has been shown to induce adaptive immune responses *in vitro*(2). In humans, DC-SIGN can be found on immature dendritic cells (DCs) and macrophages in peripheral tissues like the placenta and lung(3), as well as mature dendritic cells in lymphoid tissue(4), but not on other APC subsets, including plasmacytoid DCs or Langerhans cells(5). DC-SIGN can also be found on DCs and M2-like macrophages in tumor tissue(6,7) and on inflammatory macrophages in rheumatoid arthritis synovium(8). Interestingly, DC-SIGN expression is particularly high on monocyte-derived dendritic cells (MoDCs) and dependent on IL4(9). Although the physiological relevance of MoDCs in humans is still unclear, in mice these cells have shown to contribute to antigen presentation and T cell activation(10). Although eight DC-SIGN-related receptors are described in mice, the absence of a clear murine ortholog has hampered the *in vivo* validation of human DC-SIGN and has so far been performed with mice that express human DC-SIGN driven by the CD11c promoter(11). Subsequent targeting of antigens in this model has demonstrated the potency of hDC-SIGN on CD11c⁺ dendritic cells to internalize, process and present antigen to T cells(12,13). For example, targeting of DC-SIGN in combination with genetic depletion of regulatory T cells was sufficient to induce long-term tumor regression in B16 melanoma-bearing mice(14). A similar strategy induced high levels of antigen-specific CD8⁺ and CD4⁺ T cells,

which protected mice from *Listeria monocytogenes*(15). While it is evident that human DC-SIGN is an effective gateway to strong adaptive immunity, its expression on all CD11c⁺ cells limits its translational value as an *in vivo* model for antigen targeting.

Of the eight mouse homologues, SIGNR5/CD209a has been coined as mouse DC-SIGN (mDC-SIGN) because of similar expression patterns and localization in the genome(16). Several reports have shown mDC-SIGN to be mostly expressed by monocyte-derived dendritic cells (moDCs), which are present in steady state muscle(17) and skin(18) or develop from circulating monocytes after pro-inflammatory signals like GM-CSF(19), LPS(20) or even T cell activation(21). While mDC-SIGN⁺ monocyte-derived dendritic cells have been shown to be potent inducers of adaptive T cell immunity, it still remains unclear whether mDC-SIGN itself is able to mediate antigen uptake and presentation to T cells.

Here, we show data that support the paradigm that mDC-SIGN shares expression patterns *in vitro* and *in vivo* with hDC-SIGN, as well as functional properties, including endocytic capacity and antigen presentation to CD8⁺ and CD4⁺ T cells *in vitro*. Combining targeting of antigen to mDC-SIGN and a potent adjuvant *in vivo* generates antigen-specific CD8⁺ and CD4⁺ T cells and increased antibody responses. In particular, targeting antigen to mDC-SIGN induces significantly higher antigen-specific humoral responses.

Material and Methods

Mice

Mice transgenic for hDC-SIGN, OT-I and OT-II on the C57BL/6 background have been described previously(11,22,23). The transgenic and wild type C57BL/6 mice were bred at the animal facility of VU University (Amsterdam, Netherlands) under specific pathogen-free conditions and used at 8–16 weeks of age. Female and male mice were equally divided among groups, unless stated otherwise. All experiments were approved by the Animal Experiments Committee of the VU University and performed in accordance with national and international guidelines and regulations.

Flow cytometry facilities and reagents

All flow cytometry experiments were performed at the O2 Flow Facility at VU University (Amsterdam, Netherlands) using an X20 Fortessa flow cytometer (BD Biosciences) and ImageStreamX (Amnis Corp.) imaging flow cytometer. All antibodies were purchased from Biolegend, Miltenyi and eBioscience (ThermoFisher), specifically: anti-CD4 (Clone GK1.5), anti-CD8 (Clone H35-17.2), anti-CD11b (Clone M1/70), anti-B220 (Clone RA3-6B2), anti-Ly6C (Clone HK1.4), anti-CD11c (Clone N418), anti-NK1.1 (Clone PK136), anti-CD45 (Clone 30-F11), anti-CD3 (Clone 145-2C11), anti-CCR2 (Clone SA203G11), anti-GR1 (Clone RB6-8C5), anti-CCR7 (clone 4B12), anti-mDC-SIGN (Clone MMD3), anti-MHCII (Clone M5/114.15.2), anti-CD16/32 (Clone 93), Fixable viability dye-eFluor 780 (Thermo Fisher). OVA257-264-H2-Kb-PE tetramers were a kind gift from Dr. J.W.Drijfhout at the LUMC, Leiden, Netherlands.

Imaging flow cytometry and sample preparation

Bone marrow-derived dendritic cells (BMDCs) were cultured as described by Lutz et al. (24). Because of the high number of cells needed for image flow cytometry, no *ex vivo* isolated DCs could be used in these experiments. BMDCs were incubated with anti-mDC-SIGN:AF488 antibody (clone MMD3) for 1 hour, either on 4 °C or 37 °C. Cells were washed with PBS twice and fixed for 15 minutes using cold 4% PFA. After washing twice, the fixed cells were resuspended in PBS. Cells were analyzed on the ImageStream X100 (Amnis-Merck Millipore) imaging flow cytometer as previously described(25). A minimum of 15000 cells were acquired per sample. The internalization score was calculated as previously described(25). Briefly, cells were acquired on the basis of their area. Analysis was performed with single cells after compensation (with a minimum of 5000 cells). For standard acquisition, the 488nm laser line was set at 100 mW. Firstly, a mask was designed based on the surface of cells in the brightfield image. This mask was then eroded to exclude the cell membrane. Finally, the resulting mask was applied to the fluorescence channel. The internalization score was then calculated on this mask using the Internalization feature provided in the Ideas v6.0 software (Amnis-Merck Millipore). Internalization can be interpreted as a log-scaled ratio of the intensity of the intracellular space versus the intensity of the entire cell. Cells that have internalized antigen typically have positive scores, while cells that show the antigen still on the membrane have negative scores. Cells with scores around 0 have similar amounts of antigen on the membrane and in intracellular compartments.

Mouse tissue collection, digestion and FACS staining

Mice were sacrificed and skin-draining lymph nodes (LNs), spleen, skin and blood were obtained for further analysis. Skin-draining lymph nodes were verified by the presence of migratory DCs after 100 μ L adjuvant (25 μ g agonistic CD40 (in house 1C10) in 1:1 AddaVax (InvivoGen)) injection subcutaneously in the skin. For antigen tracking experiments, skin biopsies were taken using 8mm sterile dermal biopsy punches (KAI Medical) 2 hours after injection of fluorescently labeled antibody with adjuvant. LNs, spleen and skin were cut small using sterile scissors in 385 μ g/mL liberase TL (2WU) and incubated at 37 °C for 20 minutes. Enzymes were deactivated using ice-cold RPMI 1640 complete (10% FCS, 1% 50 U/mL penicillin, 50 μ g/mL streptomycin, HEPES/EDTA). After digestion, cells were run through a 100 μ m cell strainer and extensively washed before FACS staining. Cells were stained for 30 minutes at 4 °C using only directly labeled primary antibodies and in the presence of 1 μ g/mL anti-CD16/32 antibody. After extensive wash with PBS, labeled cells were fixed with 1% PFA at 4 °C for 15 minutes, washed and measured on the flow cytometer.

Flow cytometry analyses

Flow cytometry data was analyzed first using FlowJo analysis software. First, files were compensated using UltraComp eBeads (Thermo Fisher) microspheres labeled with the appropriate antibodies. Compensation was additionally verified using fluorescence-minus-one (FMO) controls for every single fluorochrome for every tissue type (equally pooled per group) on experimental samples. Next, first gating was performed on a stable flow (time vs. cell count), subsequently on viability dye-negative/CD45-positive cells and finally on single cells (FSC-A/FSC-H). The resulting cells were concatenated and exported per experimental group into an FCS file and uploaded to the Cytobank online analysis platform (<https://www.cytobank.org/>). Using the ViSNE module, we generate tSNE plots per tissue type based on the following input and analysis settings: all cells (concatenated) per condition used up to 300,000 total, number of iterations = 3000, Perplexity = 50, Theta = 0.5. Cells were clustered by MHCII, CD11b, CD11c, B220, NK1.1, Ly6C, GR-1, CD3 expression. Next, we identified and manually gated subpopulations as represented by the tSNE clustering analysis (Supplementary figure 2), color-coded and overlaid the subpopulations as represented in the graphs (Figure 4A and supplementary figure 2). After defining gating strategies, the individual experimental samples were similarly gated in FlowJo and statistics were exported to Graphpad Prism 6 for visualization. Histograms were generated in FlowJo by comparison of “fluorescence minus one” (FMO; all antibody minus one) or isotype antibody as negative control.

Antigen presentation assays

Bone marrow-derived dendritic cells were cultured as described by Lutz et al. (24). OTI and OTII transgenic mice were sacrificed and spleens were mechanically run through a 100 μ m cell strainer. Red blood cells were lysed using ACK lysis buffer (0.15M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) and washed before purification using MagniSort Mouse CD4- or CD8-negative isolation kits according to manufacturer's instructions (eBioscience/Thermo Fisher). Purified CD4⁺ and CD8⁺ T cells were labeled using 2 μ M CFSE and counted before co-culture. BMDCs and purified T cells were co-cultured for 3 days at 37 °C, stained and measured on a X-20 Fortessa flow cytometer. To avoid overestimation of CFSE-based proliferation results (26), we adopt the %responding cells metric. Results are calculated and represented as percentage responding cells ('calculated cells at the start of culture'/'number of cells that went into division'*100). Total # of cells at start of culture = #G0+(#G1/2)+(G2/4)+(G3/8)+ (#G4/16)+(G5/32)+(G6/64). Number of cells that went into division = 'Total # of cells at start of culture' - G0.

Generation of ovalbumin-coupled antibody

In this study we have used two clones of mDC-SIGN-binding antibodies (MMD2 and MMD3) with identical properties to visualize the receptor and investigate functional characteristics of the mDC-SIGN molecule (27). Anti-DC-SIGN (clone AZN-D1, clone MMD2) and IgG2c (clone 6.3; SouthernBiotech) antibodies were conjugated to ovalbumin (OVA; Calbiochem, Darmstadt, Germany) using the cross-linking agent sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate or Alexa Fluor 488 NHS Ester (20000; Thermo Fisher) according to the manufacturer's protocol (Pierce). Antibody-conjugates were separated from reaction-reductants using PD-10 desalting columns (Pierce, Rockford, USA). The concentration of OVA and antibody was determined using the bicinchoninic acid assay (Pierce) and ELISA. The products were tested for endotoxins using the Limulus Amebocyte Lysate assay and a level of <0.125 EU/mL was deemed acceptable.

Vaccination

Mice were subcutaneously injected with endotoxin-free 25 μ g anti-mDC-SIGN:OVA or anti-mDC-SIGN:AF488 with 25 μ g agonistic CD40 antibody (in house, clone 1C10) in 1:1 PBS/AddaVax emulsion according to manufacturer's instructions (InvivoGen) in a maximum volume of 100 μ L. For functional readouts, spleens and blood were collected 7 days after vaccination. For antigen tracking experiments, organs were harvested 2 hours and 12 hours after vaccine injection. For mDC-SIGN phenotyping experiments, C57BL/6 mice were sacrificed and organs harvested as described.

OVA-specific antibody determination

To determine the antigen-specific antibody response to the ovalbumin, NUNC Maxisorp 96-well plates (Thermo Fisher) were coated with 10ug/ml purified ovalbumin (Sigma-Aldrich) for 24 hours at 4 °C in coating buffer in PBS. Next, plates were washed extensively with PBS/Tween 0,05% and additionally blocked with 1% PBS/BSA. A dilution range of serum, obtained from vaccinated mice (on day 7 after vaccination) through a heart puncture, was incubated over night at 4 °C. After washing, samples were incubated with anti-mouse IgG-biotin (and anti-IgG1, 2, 3 isotypes) antibodies for 1 hour at RT and after wash incubated with HRP-conjugated streptavidin for 1 hour at RT. Then after washing, the ELISA plate was developed using TMB substrate buffer. Reaction was stopped when properly developed using 2N H2SO4 and extinction was measured at 450nm using an iMark microplate reader (Bio-Rad). Serum dilution of 1:400 showed the most consistent and reproducible signal to noise ratio. All samples were normalized with PBS as blanco. Secondary antibodies (1:2000) used: IgG1 115-065-205 (Jackson ImmunoResearch), IgG2a m32215 (Invitrogen), IgG2b ab97248 (Abcam), IgG3 1100-08 (ITK), IgM 62-6840 (Zymed), IgG 315-065-006 (Dianova), Streptavidin-HRP p0397 (Dako).

Statistics

Statistics were performed using GraphPad Prism 6 software. For the comparison of two groups a student's t test was used. For more than two groups a two-way analysis-of-variance (ANOVA) was used followed by a Tukey posthoc analysis to compare means between two groups. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, data represented as mean+SEM.

Results

First, to study the functional characteristics of mDC-SIGN, we examined its expression on cultured dendritic cells *in vitro* by flow cytometry. We generated bone marrow-derived dendritic cells (BMDCs), as previously described(24), using bone marrow from hDC-SIGN transgenic mice(11). GM-CSF-cultured BMDCs roughly generate two populations of antigen presenting cells distinguished by CD11c and MHC class II expression, GM-DCs (CD11c⁺MHCII^{high}) and GM-Macs (CD11c⁺MHCII^{int})(28). Both classically GM-CSF-cultured bone marrow-derived dendritic cells and macrophages showed clear expression of mDC-SIGN and hDC-SIGN (Figure 1). No difference in mDC-SIGN expression between WT and hDC-SIGN transgenic was observed, while hDC-SIGN was absent on wild type BMDCs, as expected (data not shown). Interestingly, mDC-SIGN is higher expressed on GM-Macs compared to GM-DCs cultured from bone marrow precursors.

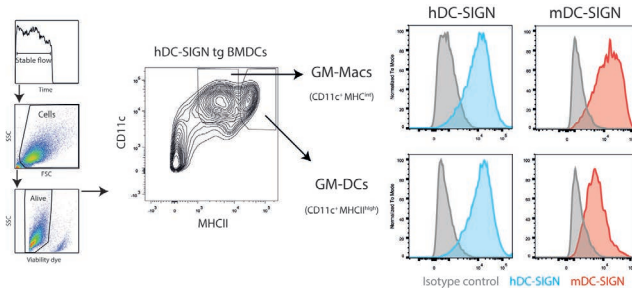


Figure 1 | Mouse DC-SIGN is expressed by GM-DCs and GM-Macs cultured *in vitro*. Both GM-CSF-cultured (differentiation for 7 days) bone marrow-derived macrophages (GM-Macs) and dendritic cells (GM-DCs) express mDC-SIGN. Data is representative of 2 individual experiments.

To investigate whether mDC-SIGN behaves as an internalizing receptor like hDC-SIGN, we determined the endocytic capacity of BMDCs by imaging flow cytometry. Given the sequence similarity of the different family members in the DC-SIGN family, we selected a set of monoclonal antibodies that have been previously demonstrated to specifically recognize either mDC-SIGN (MMD2/MMD3) or hDC-SIGN (AZN-D1) (4,20,27). Fluorescently-labeled mDC-SIGN antibody (α -mDC-SIGN-AF488) recognizes mDC-SIGN, which is Fab-dependent as it cannot be blocked by pre-incubation with IgGs or FC-block for 30 min (Figure 2A), as previously described(20). Using imaging flow cytometry, we can discriminate between membrane-bound fluorescence and intracellular fluorescence and thereby follow internalization of the receptor (Figure 2B). To analyze the capacity of mDC-SIGN antibody to internalize *in vitro*, we incubated GM-CSF cultured BMDCs with anti-mDC-SIGN antibody at either

4 °C or 37 °C for an hour. We observed clear internalization of the fluorescently-labeled mDC-SIGN antibody after 1 hour incubation at 37 °C compared to 4 °C, when cells are metabolically inactive (Figure 2C; red line and blue line, respectively). Interestingly, no obvious accumulation of fluorescent signal is observed when the fluorescently-labeled antibody is continually present for an hour (Figure 2D). This suggests that mDC-SIGN is either not recycled within this time window to accumulate more antibody intracellularly, or the degradation of antibody is balanced by continuous uptake. To examine this, we tested whether mDC-SIGN was available on the membrane for antibody binding after 1 hour of internalization with mDC-SIGN targeting antibody. Pulse-chase experiments revealed quick degradation of the fluorochrome after pulse (Figure 2E; blue line), which could not be blocked by pre-incubation with the same antibody (black line). When mDC-SIGN-AF488 fluorescently-labeled antibody was incubated for 1 hour and allowed to internalize at 37 °C, unbound membrane-bound mDC-SIGN molecules were still available for staining with a second mDC-SIGN-eFluor660 antibody (Figure 2F, green lines and red lines respectively). Also, the secondary antibody was internalized and degraded in a similar fashion to the mDC-SIGN-AF488 antibody. The fact that mDC-SIGN was apparently available for antibody binding after 1 hour of internalization shows that mDC-SIGN is either recycled or newly synthesized. Taken together, these data suggest that mDC-SIGN has endocytic capacity as previously described for hDC-SIGN(29), but quickly resurfaces on the cell membrane for binding and uptake.

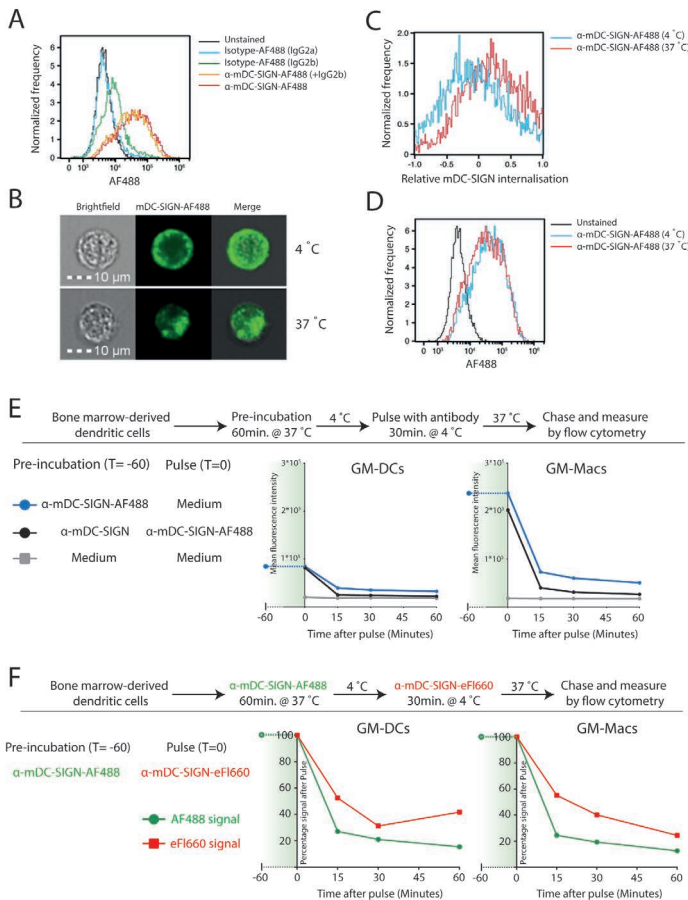


Figure 2 | Mouse DC-SIGN (mDC-SIGN) on GM-DCs and GM-Macs is a quickly internalizing receptor for antigen processing.

A. Anti-mDC-SIGN binding to bone marrow-derived dendritic cells (BMDCs) cannot be blocked by isotype-IgG preincubation.

B. Example of ISX Image Stream data of mDC-SIGN-AF488 binding and internalization on BMDCs.

C. Upon binding, mDC-SIGN is quickly internalized at 37 °C for 1 hour. **D.** Anti-mDC-SIGN-AF488 fluorescence does not increase after 1 hour at 37 °C, suggesting either a balance in uptake and degradation or an absence of continued uptake. Experiments representative of 2 individual experiments.

E. Pulse-chase experiments show that pre-incubation of BMDCs with unlabeled anti-mDC-SIGN (clone MMD2) for 1 hour at 37 °C does not abrogate pulse binding of labeled anti-mDC-SIGN-AF488 (clone MMD3). Additionally, fluorescent signal is quickly reduced as the antibody is internalized and degraded in both GM-DCs and GM-Macs. **F.** BMDC labeling 1 hour with anti-mDC-SIGN-AF488 (clone MMD3) prior to pulse staining with anti-mDC-SIGN-eFluor660 (clone MMD3)

shows similar availability of membrane-bound mDC-SIGN molecules after internalization by the first AF488-labeled antibody.

Since hDC-SIGN is able to route internalized antigen to MHC class I and II complexes for presentation to T cells(5), we investigated the antigen presenting capacity of mDC-SIGN⁺ dendritic cells *in vitro*. GM-CSF cultured hDC-SIGN transgenic BMDCs express both mDC-SIGN and hDC-SIGN (Figure 1), allowing the comparison between these receptors using the same bone marrow culture. hDC-SIGN BMDCs pulsed with mDC-SIGN:OVA or hDC-SIGN:OVA targeting antibody were able to internalize, process and present antigen to OTI CD8⁺ or OTII CD4⁺ T cells (Figure 3). Additionally, the TLR4 agonist and known inducer of cross-presentation to CD8⁺ T cells(30), monophosphoryl lipid A (MPLA), significantly boosted antigen presentation to CD4⁺ and CD8⁺ T cells, with a more pronounced effect on CD8⁺ T cells (Figure 3). The antigen presentation capacity of mDC-SIGN using wild type BMDCs resulted in comparable results, whereas T cell activation by hDC-SIGN:OVA was abolished in WT BMDCs lacking hDC-SIGN (Supplementary Figure 1). Hence, both mDC-SIGN and hDC-SIGN are capable of antigen presentation to CD4⁺ T cells and cross-presentation to CD8⁺ T cells *in vitro*, which can be boosted by TLR4 activation through MPLA. These data clearly support mDC-SIGN as an endocytic receptor for antigen presentation to T cells, similarly to hDC-SIGN.

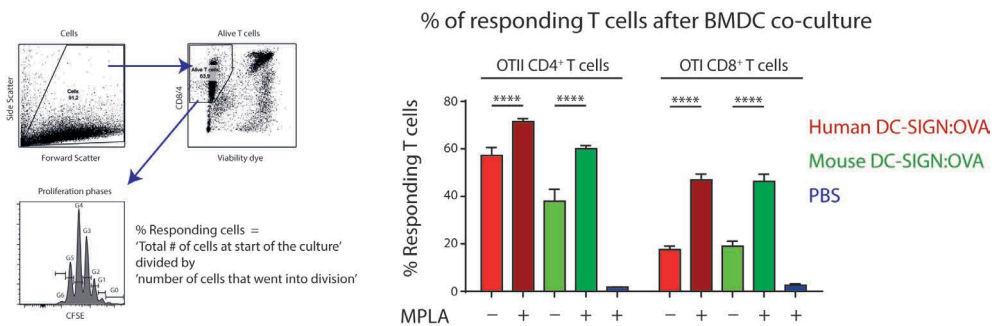


Figure 3 | Antigen targeting to mouse- and human DC-SIGN on BMDCs leads to internalization, processing and presentation to antigen-specific CD8⁺ and CD4⁺ T cells. GM-CSF cultured hDC-SIGN BMDCs pulsed with 1 μ g/ml α -mDC-SIGN:OVA or α -hDC-SIGN:OVA (1hr 37°C, with Fc block) presents processed antigen to CFSE-labeled OTI CD8⁺ or OTII CD4⁺ T cells after 3 days of co-culture. Also, the TLR4 ligand, MPLA consistently boosts antigen presentation. T cell proliferation is represented as percentage responding cells ('calculated cells at the start of culture'/'number of cells that went into division'*100). Data represented as mean \pm SEM, co-cultured in triplicates (ANOVA with Tukey post-hoc ****p<0,0001), representative of 3 individual experiments.

Next, we aimed to explore mDC-SIGN expression on the major immune subsets in the skin, spleen, lymph node and blood of C57/Bl6 mice. Using a 12-color flow cytometry panel including directly labeled antibodies against CD45, CD3, B220, NK1.1, Ly6C, GR-1, CD11b, CD11c, MHCII, CCR2, mDC-SIGN and a viability dye, we could dissect the major immune population present in blood, spleen, skin, and skin-draining lymph node (ie. lateral inguinal LN). To distinguish the populations, we applied tSNE unsupervised clustering as previously described(31), using the online analysis platform Cytobank. The output in Figure 4 represents all alive CD45⁺ cells with high dimensional data in a two-dimensional plot (tSNE1 vs tSNE2). Cells that are similar in marker expression are clustered together in space. This approach prevented us from overlooking subpopulations of immune cells in the tissue, while developing proper manual gating strategies (Supplementary figure 2). Additionally, by manually gating we could assimilate individual subsets into a clear immune composition of the tissue (Figure 4). The immune composition of the skin seems to be largely dominated by skin macrophages/monocytes (CD11b⁺Ly6C⁺GR-1⁻ (orange)), dermal T cells (NK1.1⁻CD3int (brown)) and CD11c⁺ APCs (CD11b⁺Ly6C⁺GR-1⁻CD11c⁺MHCII⁺ (green)). Interestingly, the CD11b⁺ DCs and the CD11c⁺ APCs could only be distinguished by CD11c expression, and could not be further subdivided based on the markers used in this panel. Hence, they are not distinctly separated in the tSNE analysis, suggesting that these cell types are similar based on the markers used. The immune composition of lymph nodes, spleen and blood is mainly dominated by lymphoid cells like B-, T- and NK cells (gray, purple and pink, respectively). Subsequent gating showed low but clear mDC-SIGN expression mostly on Ly6Chigh

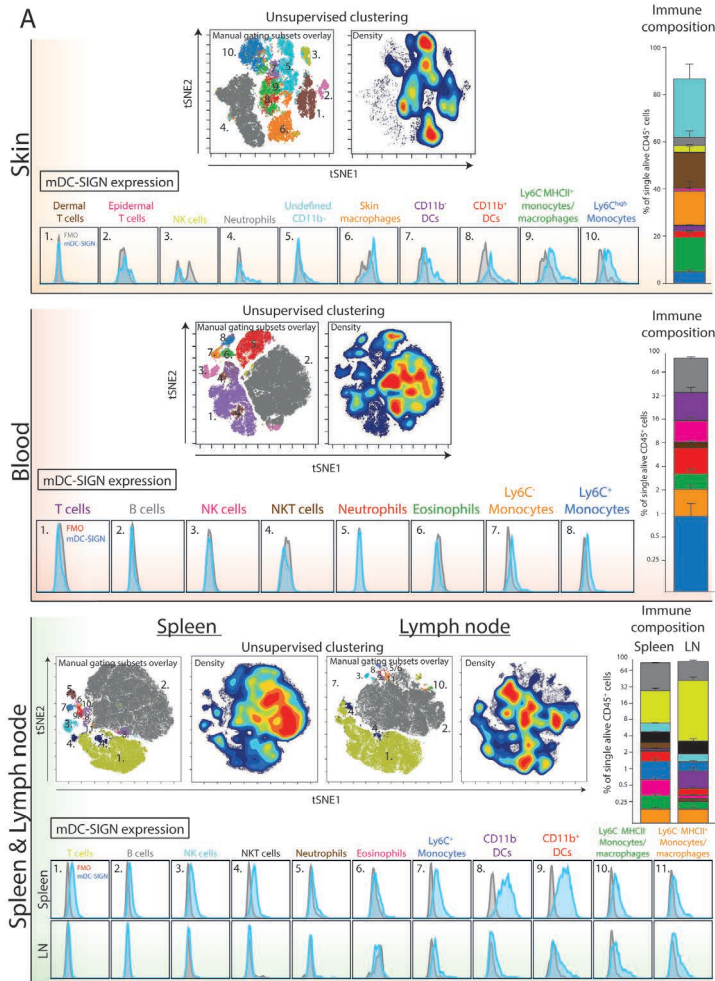
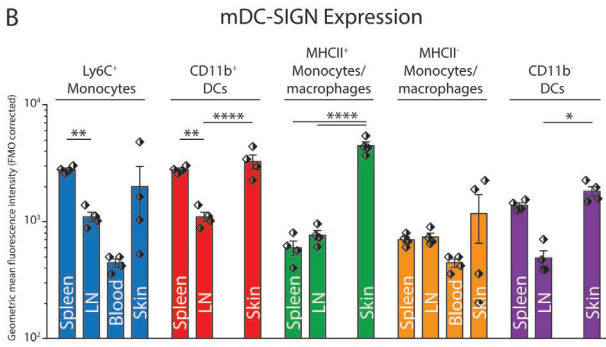


Figure 4 | Unsupervised clustering (tSNE) of total CD45⁺ cells distinguishes organ-specific immune cell subsets expressing mDC-SIGN. A. Unsupervised clustering (tSNE) of alive single CD45⁺ cells based on high dimensional flow cytometry into two dimensions allows the distinction of immune subsets and reconstruction of cell frequency (density plot) within the immune composition. Expression of mDC-SIGN (blue histogram) is generally expressed by monocytes/macrophages in the skin and spleen. Dendritic cells in the spleen express clear levels of mDC-SIGN, but expression is less pronounced in the skin-draining lymph nodes. Data in the bar graphs represented as mean \pm SEM (N=4, representative of 2 individual experiments). B. (next page) mDC-SIGN is highly expressed on myeloid cells and in particular monocytes/ macrophages and DCs in the skin. Also, expression is high on CD11b- and CD11b⁺ DCs in the spleen, compared to the lymph node.

monocytes (CD11b⁺Ly6C^{high}GR-1^{int}), Ly6C⁺ monocytes/macrophages (CD11b⁺Ly6C⁺) and CD11b⁺ and CD11b⁻ dendritic cells (MHCII⁺CD11c⁺) in the skin and spleen (Figure 4A). Additional gating strategies to identify splenic CD11b⁻ and CD11b⁺ DCs (Figure 4; in purple and red) resulted in similar expression levels (Supplementary figure 3). Lymphoid cell types and granulocytes like neutrophils and eosinophils were generally devoid of mDC-SIGN, although T-, NK- and NKT cells showed very low levels of mDC-SIGN staining in the spleen. Expression on DCs was clearly present in the spleen, but significantly lower in the lymph node, while mDC-SIGN was absent on immune cells in blood or low on blood monocytes (Figure 4B).

To investigate the potential of mDC-SIGN⁺ APCs to facilitate adaptive immunity, we injected fluorescently labeled mDC-SIGN targeting antibody and isotype control antibody subcutaneously in the skin combined with adjuvant (agonistic CD40 in MF59/AddaVax emulsion)(32). Using similar gating strategies as previously shown, injected mDC-SIGN targeting antibody can be found on mDC-SIGN⁺ APCs in the skin within 2 hours after injection (Figure 5A). Notably, fluorescent IgG2c isotype control antibody was mostly bound by CD11b⁺Ly6C⁺CD11c⁺MHCII⁺ monocytes/macrophages and to some extent by CD11b⁺ dendritic cells, but not CD11b⁻ dendritic cells and monocyte-derived dendritic cells (Figure 5A). Of note is the high level of signal from targeted APCs *in situ* compared to *ex vivo* stained skin APCs, which



likely reflects the rapid turnover rate of the mDC-SIGN molecule, leading to signal accumulation in the 2 hours after injection of the antibody. To distinguish between LN-homing dendritic cells and antigen presenting cells unable to migrate to LNs, we stained CCR7 on isolated APCs, 2 hours after subcutaneous antibody injection. We could verify CCR7 expression on all DC subsets, including CD11b⁺Ly6C⁺MHCII⁺ monocyte-derived dendritic cells, although expression levels were low (Figure 5B). To analyse the fate of the mDC-SIGN targeting antibody in the draining lymph nodes, we isolated skin-draining lymph nodes 12 hours after subcutaneous injection of fluorescently labeled mDC-SIGN targeting antibody and analyzed the content of targeted dendritic cells. CD11b⁺ dendritic cells, monocyte-derived dendritic cells and CD11b⁺ dendritic cells contained significantly more skin-injected mDC-SIGN targeting antibody compared to IgG control antibody (Figure 5C). Direct targeting of injected antibody to the skin-draining lymph node did not occur within 2 hours after injection (Supplementary figure 4), suggesting that these cells are derived from the periphery and are not labeled through direct drainage to the lymph node. Importantly,

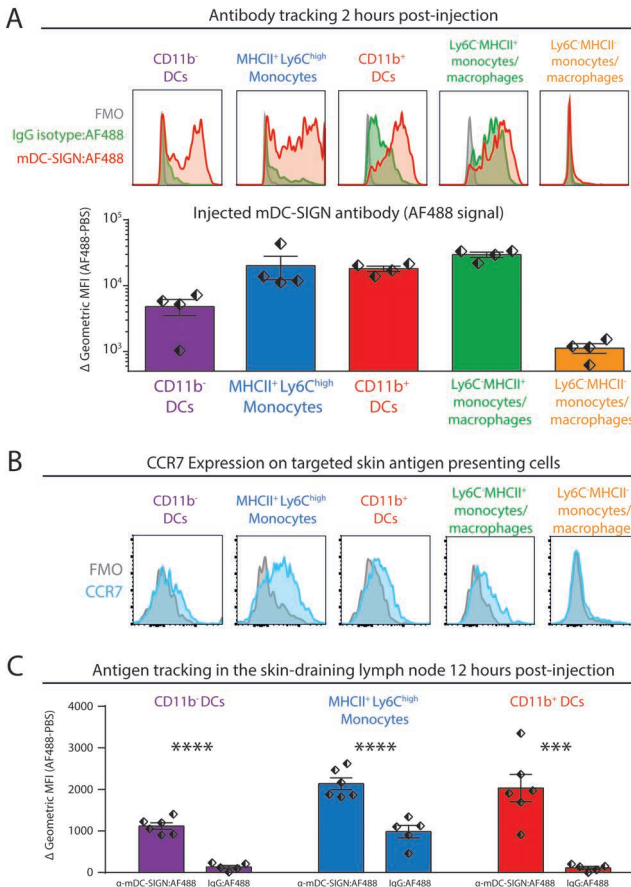


Figure 5 | Subcutaneous injection of anti-mDC-SIGN antibody targets skin mDC-SIGN⁺ APCs primarily in the skin A. 2 hours after subcutaneous injection of fluorescently labeled anti-mDC-SIGN in adjuvant (MF59/ AddaVax with agonistic anti-CD40 antibody) shows targeting of skin APCs. mDC-SIGN antibody shows particular targeting of MHCII⁺Ly6C^{high} monocytes, CD11b⁺ and CD11b⁺ dendritic cells. Red/Green = fluorescent signal, Grey = FMO negative control. B. Expression of CCR7 on *ex vivo* isolated skin-derived antigen presenting cells 2 hours after subcutaneous injection of anti-mDC-SIGN antibody. C. Anti-mDC-SIGN-AF488 antibody-labeled APCs can be found in the skin-draining lymph node 12 hours after injection. Data represented as mean + SEM, ANOVA with Tukey post-hoc ***p<0,001, ****p<0,0001, representative of 2 individual experiments.

labeling was the most proficient in CD11b⁺ dendritic cells and monocyte-derived dendritic cells (Figure 5C).

To determine whether *in vivo* targeting of antigen to mDC-SIGN induces adaptive immunity, subcutaneous vaccination using mDC-SIGN targeting antibody-coupled to ovalbumin (OVA) protein, with AddaVax-containing adjuvant was assessed. After 7 days, spleens and serum from s.c. vaccinated mice were collected to measure the generation of the adaptive immune response. A single dose of adjuvanted mDC-SIGN targeting antibody resulted in the generation of *de novo* antigen-specific CD8⁺ T cells (Figure 6A) and CD4⁺ T cells (Figure 6B). Antigen (OVA)-specific CD8⁺ T cells obtained high polyfunctionality as measured by intracellular cytokine staining after peptide re-stimulation (Figure 6A and 6B). Indeed, up to 50% of antigen-specific CD8⁺ T cells are TNF α , IFN γ and IL-2 triple-producers upon antigen-specific restimulation. Interestingly, while targeting mDC-SIGN did not show significant differences in the quantity or quality (ie. cytokine production) of the T cell responses compared to the whole protein ovalbumin, clear differences were observed in the humoral response in mDC-SIGN-OVA vaccinated mice 7 days after vaccination. OVA-specific IgG antibody titers (IgG1, IgG2a/b/c and IgG3) after 7 days were particularly high when the antigen was targeted to mDC-SIGN (Figure 6C). Notably, the bias for B cell responses was not dependent on Fc-mediated uptake in the skin as IgG2c-OVA contributed significantly to the induction of T cell responses, while significantly less OVA-specific antibody responses were measured (Supplementary Figure 4). As such, mDC-SIGN⁺ APC targeting in the skin induces strong humoral responses while retaining cellular responses, further potentiating vaccination potential.

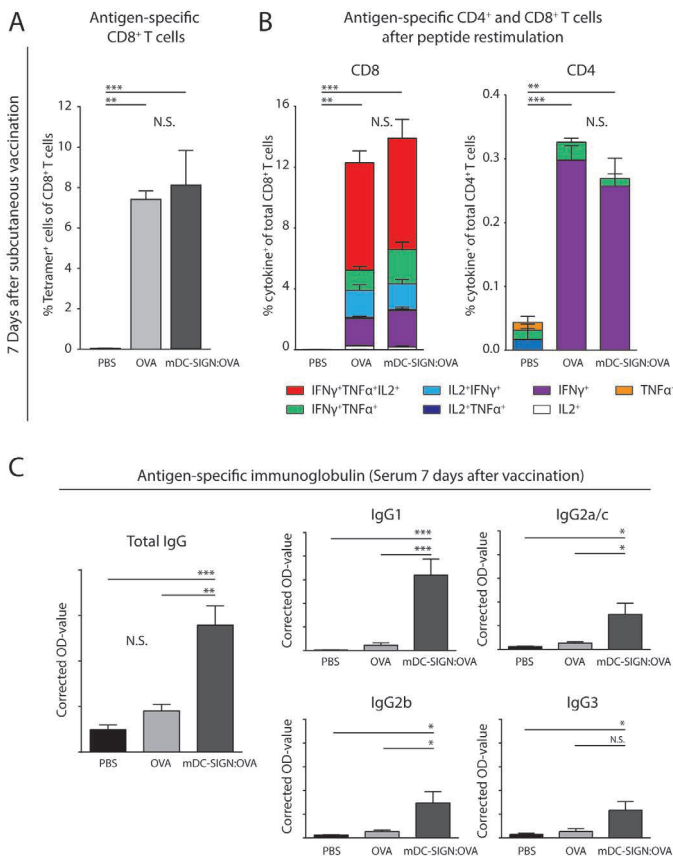


Figure 6 | One subcutaneous dose of mDC-SIGN-OVA in adjuvant leads to antigen-specific CD8⁺ and CD4⁺ T cell responses and an enhanced isotype-switched OVA-specific antibody response. A. Antigen-specific CD8⁺ T cells in splenocytes 7 days after vaccination as measured by H2-kb-SIINFEKL tetramer staining. B. T cell restimulation through incubation with cognate antigen and intracellular cytokine staining reveals polyfunctional antigen-specific CD8⁺ and CD4⁺ T cell responses. C. Antigen-specific antibody capture ELISA reveals antigen-specific immunoglobulin production in the serum of mice vaccinated with mDC-SIGN-OVA (7 days after vaccination; 1:400 serum dilution). All data represented as mean \pm SEM (N=5 per group). Two-way ANOVA with Tukey post-hoc; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Graphs are representative of 2 individual experiments.

Discussion

The C-type lectin receptor DC-SIGN has been described as a crucial innate immune receptor involved in a plethora of immunological processes, including the recognition of pathogen-derived ligands and self-glycoproteins, intracellular signaling, antigen processing and presentation, and activation of T cells(1). However, research on its physiological role *in vivo* has been hampered because the lack of a true ortholog in the murine genome(16). Nonetheless, CD209a (also known as SIGNR5) has been coined as mouse DC-SIGN (mDC-SIGN) because of its overlapping expression patterns and localization in the genome. Here, we show expression of mDC-SIGN on GM-CSF cultured bone-marrow derived macrophages (GM-Macs) and dendritic cells (GM-DCs) *in vitro*. Interestingly, a detailed study on the identity and transcriptome of GM-DCs and GM-Macs has suggested their similarity with *in vivo* migratory DCs and skin-resident monocyte-derived DCs/macrophages, respectively(28). This appears to be in line with our *in vivo* data that mDC-SIGN can be found on both skin-resident DCs and skin-resident monocytes. More importantly, *in vivo* GM-CSF-dependent mDC-SIGN⁺ Mo-DCs seem to arise from specific FcγIIIR*MHCIIR*mDC-SIGN⁺ monocytes(19). Nonetheless, while human DC-SIGN can be found on GM-CSF cultured MoDCs and skin-resident CD14⁺ macrophages, the expression of human DC-SIGN⁺ on migratory DCs remains to be demonstrated, suggesting a possible discrepancy in expression patterns between mouse and human DC-SIGN.

Expression of mDC-SIGN seems to be most pronounced on antigen presenting cells in organs like the spleen and skin, where pathogens are most likely to be encountered. This may suggest an endogenous role for mDC-SIGN as pattern recognition receptor, similar to human DC-SIGN. However, human DC-SIGN functions as an important pattern recognition receptor via its carbohydrate recognition domain, allowing the binding of particular sugar-structures on pathogens like HIV-1, Ebola virus, *Mycobacterium tuberculosis*, *Candida albicans*, *Schistosoma mansoni*, and *Helicobacter pylori*(33). In contrast, mDC-SIGN does not seem to share the exact ligand-specificity as human DC-SIGN (34), but has not been carefully studied in detail. Still, fucosylated Lewis x antigen, a known human DC-SIGN ligand, mediated suppression and tolerance to transplantation through mDC-SIGN⁺ monocyte-derived macrophages(35). Additionally, a recent study on mDC-SIGN functioning during Schistosome egg infection revealed the capacity of mDC-SIGN to signal via Raf-1 depending on its carbohydrate recognition domain and affect DC functioning(36). Therefore, studies aimed at investigating DC-SIGN functioning *in vivo* that rely on recognition of specific glycan structures should take the ligand binding specificity into consideration, as well as the capacity of mDC-SIGN to affect intracellular signaling. Interestingly, among the other mouse homologs, CD209b (or SIGNR1) has been most widely investigated and shares glycan-binding specificity, including Lewis antigens, with human DC-SIGN(1). Also, the glycan-binding properties of CD209b are vital to the immunological response to *Candida albicans*(37), influenza(38) and pneumococcal polysaccharides(39). However, its expression by subcapsular macrophages in lymph nodes and marginal zone macrophages more resembles the human DC-SIGN homologue L-SIGN(40).

Regardless of endogenous ligand specificity, we provide evidence that mDC-SIGN is an internalizing receptor capable of internalizing antigen, resulting in antigen presentation to T cells. However, where hDC-SIGN has been shown to be a slow-recycling receptor(29), mDC-SIGN shows quick recovery and membrane expression after internalization. Therefore, the mode of mDC-SIGN molecule membrane homeostasis is differently regulated compared to hDC-SIGN. A current limitation of the study is the use of antibodies to target a receptor, which can add Fc-mediated effects to the experimental outcome of the results. However, since many murine SIGNR molecules exist with overlapping ligand specificity, investigating the function of mDC-SIGN *in vivo* can currently only be done using highly specific antibodies. Nonetheless, our data support the paradigm that mDC-SIGN displays functional homology to hDC-SIGN, sharing expression *in vitro* and *in vivo*, endocytic capacity and antigen presentation to CD8⁺ and CD4⁺ T cells. This is of particular interest, since C-type lectins like DC-SIGN have been implicated in T cell functioning in humans(41). In mice, mDC-SIGN⁺ monocyte-derived dendritic cells and macrophages have been shown to control T cell-mediated responses to transplantation tolerance(35), cerebral malaria(42), murine schistosomiasis(43), LPS-

induced systemic infection(20) and experimental colon inflammation(44). Also, mDC-SIGN targeting antibody to skin mDC-SIGN⁺ APCs and skin mDC-SIGN⁺ monocytes induced antigen-specific CD8⁺ and CD4⁺ T cell responses. It remains to be defined which subset migrates to the draining lymph node to contribute to T cell activation, especially since monocyte-derived dendritic cells are not assumed to possess high migratory potential out of peripheral tissues(45). Nonetheless, targeting mDC-SIGN led to high antigen-specific antibody responses, suggesting a potency for mDC-SIGN⁺ APCs to induce germinal center B cell responses. Notably, while conventional DCs initiate T cell responses, monocyte-derived DCs specifically boost the T follicular helper program that is needed to induce potent germinal center responses *in vivo*(46). Human monocyte-derived dendritic cells have been shown to direct follicular helper T cell differentiation and subsequent T-cell-dependent IgG production by B cells *in vitro* through DC-SIGN(47). Therefore, it is possible that through targeting antigen to mDC-SIGN the mDC-SIGN⁺ monocyte-derived dendritic cells provide additional Tfh programming and subsequent B cell responses. Alternatively, targeted deletion of CD11b⁺ dendritic cells, a mDC-SIGN⁺ DC subset in the murine skin readily targeted in the presented vaccination strategy, has shown to reduce humoral responses to vaccination(48). As such, the specific contribution of specific mDC-SIGN⁺ APC subsets remains to be defined.

It is this potency by DC-SIGN⁺ cells to control adaptive immunity that has stimulated research that targets this receptor for therapeutic purposes. Indeed, recent progress has been made in using human DC-SIGN to target tumor-associated antigens to dendritic cells for T cell activation(13,14,49,50). Indeed, in the hDC-SIGN:CD11c humanized mice, targeting OVA to hDC-SIGN on dendritic cells elicited antigen-specific CD8⁺ T cell responses capable of eradicating OVA-expressing melanoma tumors(14). Since hDC-SIGN is expressed by all CD11c⁺ cells in this model and does not reflect the natural situation in humans, targeting antigen to mDC-SIGN provides a novel method to investigate hDC-SIGN-generated adaptive immune responses. Most notably, targeting monocyte-derived dendritic cells through DC-SIGN could contribute to vaccine efficacy through the generation of antibody responses. The hDC-SIGN transgenic mouse model also does not express hDC-SIGN on cells that do not express CD11c, including CD11c⁻ APC and Ly6C⁺MHCII⁺ monocytes, which express mDC-SIGN. In conclusion, using mDC-SIGN as a hDC-SIGN homologue will allow further study of DC-SIGN-initiated adaptive immune responses in the context of a complex immune system *in vivo* and will aid preclinical DC-SIGN-targeting vaccination strategies. Notwithstanding, since the mDC-SIGN receptor internalization/resurfacing characteristics and ligand specificity is different from hDC-SIGN, there is still a need to investigate the human DC-SIGN molecule in its functional tetrameric form and under its proper genomic control *in vivo*.

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Conflict of interest

The authors state no conflict of interest.

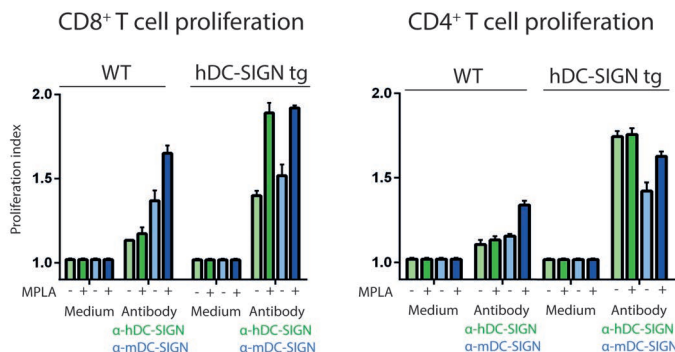
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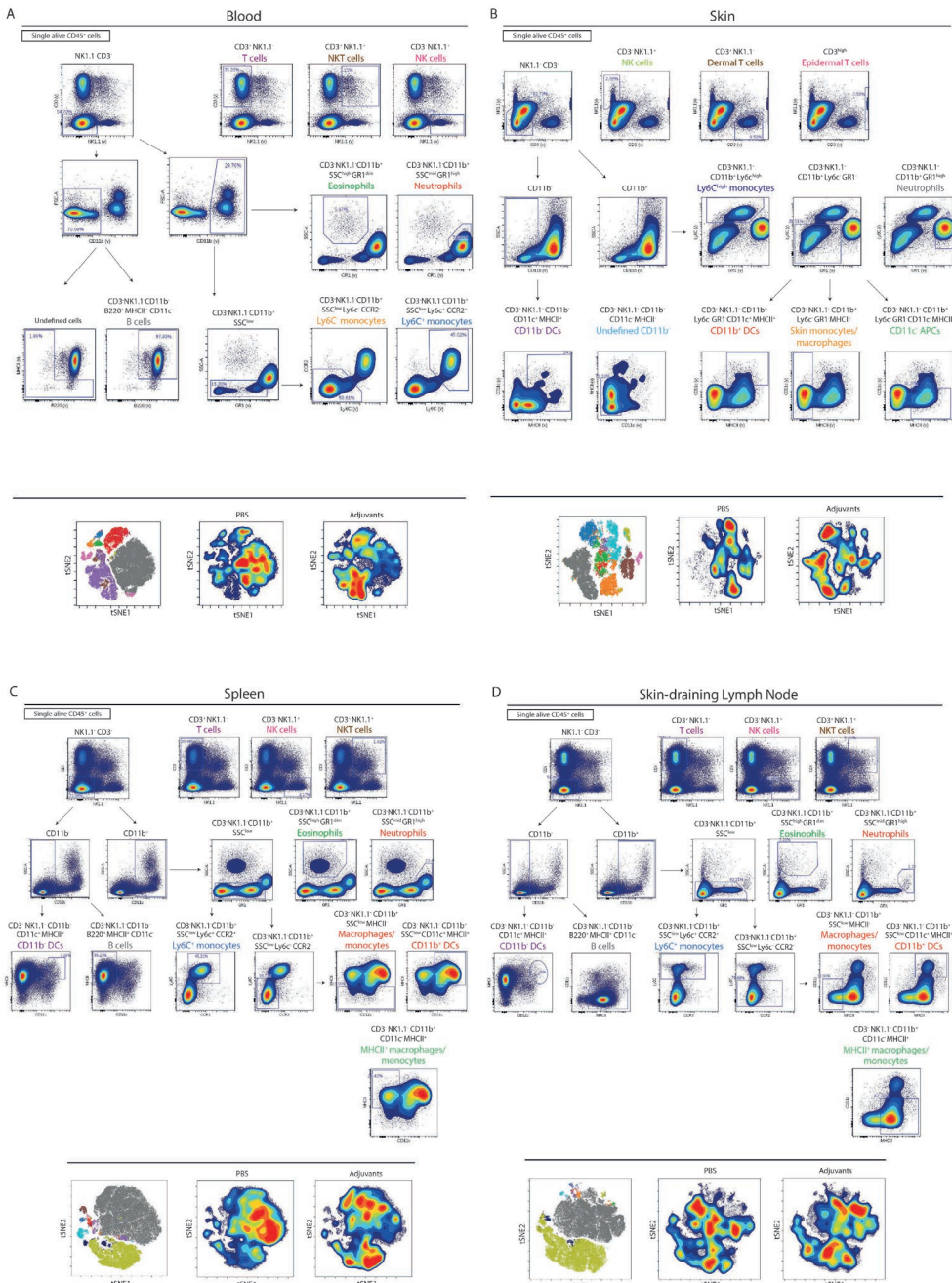
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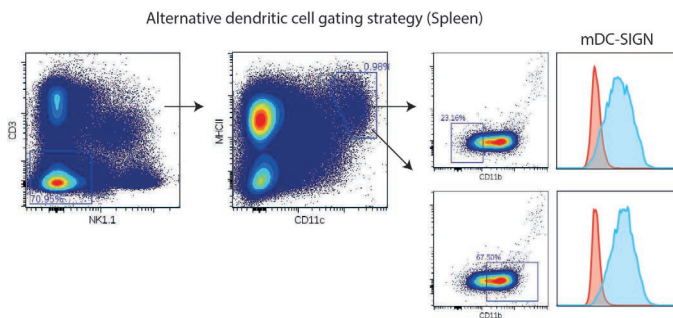
Supplementary figures



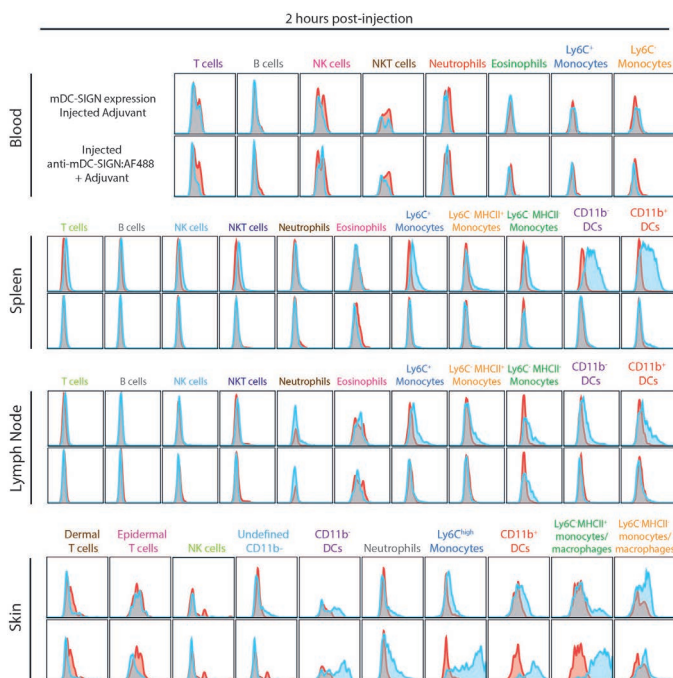
Supplementary figure 1 | Antigen targeting to mouse- and human DC-SIGN on BMDCs induces proliferation in antigen-specific CD8⁺ and CD4⁺ T cells. CD8 and CD4 T cell proliferation by hDC-SIGN was practically abolished in WT BMDCs.

All statistical graphs are generated from manually gated subsets from individual mouse samples as show here. Organs include blood (A), skin (B), spleen (C) and skin-draining lymph node (D). The tSNE graphs are generated using both homeostatic and vaccinated conditions as shown here.

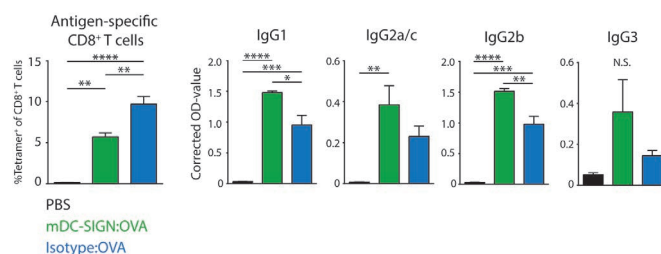




Supplementary Figure 3 | Alternative gating strategies for splenic DCs shows mDC-SIGN expression on CD11b⁺ and CD11b⁻ splenic DCs. Classical DC gating strategies by first gating on CD3⁺NK1.1⁻, then CD11c⁺MHCII⁺ cells, shows similar expression of mDC-SIGN on CD11b⁻ and CD11b⁺ splenic DCs



Supplementary figure 4 | Subcutaneous injection of mDC-SIGN antibody primarily targets skin-resident APCs. After 2 hours of subcutaneous vaccination with fluorescently labeled anti-mDC-SIGN in adjuvant (MF59/AddaVax with agonistic anti-CD40 antibody) shows targeting of skin APCs. Upper row represents mDC-SIGN expression 2 hours after adjuvant injection without labeled antibody, while the lower row shows fluorescent signal 2 hours after adjuvant injection with labeled antibody. Blue = fluorescent signal, Red = FMO negative control



Supplementary Figure 5 | Vaccination with IgG2c and mDC-SIGN antibodies shows mDC-SIGN-specific bias towards humoral responses compared to IgG2c control antibody. Seven days after subcutaneous injection of 25ug anti-mDC-SIGN:OVA antibody or IgG2c:OVA isotype control antibody

(+agonistic CD40 in 1:1 MF59/AddaVax) shows induction of antigen-specific CD8⁺ T cell responses and humoral responses. Targeting mDC-SIGN significantly generates more antigen-specific antibodies, while the isotype control generates more antigen-specific CD8⁺ T cells.

